Lucigen®
Simplifying Genomics

NxSeq® Long Mate Pair Library Kit

Generating Libraries using 2-8 kb inserts

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MA 160 Rev. G

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user, especially the specimens to be amplified, are of the highest quality. Please follow the instructions carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications.

Thank you.
Lucigen Technical Support:
Email: techserv@lucigen.com
Phone: (888) 575-9695

Product Guarantee: Lucigen guarantees that this product will perform as specified for one year from the date of shipping.

Product Description

The NxSeq® Long Mate Pair Library Kit is designed to generate mate pair librariesfor sequencing on Illumina platforms. When combined with fragment library sequencing data, mate pair library sequences enable superior genome assembly, closure, and finishing. Applications include de novo genome assembly, chromosomal rearrangement detection, haplotyping, and BAC sequencing.

There are two protocols available for this product:

- This protocol (MA160) uses a fast and easy gel-free bead clean up step for insert size selection, and is recommended for spanning repeats <8 kb.

- MA162 uses a gel-based clean up step for insert size selection and is recommended for spanning repeats from 10-20 kb.

For complex genomes, such as plant or animal genomes, we recommend constructing multiple mate pair libraries ranging from 2 - 20 kb, which would use both protocols. In most cases, very complex genomes will also benefit from a separate product, the pNGS Fosmid NxSeq 40 kb Mate Pair Library Kit (available separately).

Please contact custserv@lucigen.com with any questions.

Workflow

Using this method, genomic DNA is sheared to the desired size, end repaired, A-tailed, and ligated to adaptors. The insert is size-selected and ligated to a unique coupler that contains encrypted Chimera Code™ sequences. The circularized inserts are then treated with exonuclease to remove unwanted linear DNA and then digested with a selection of endonucleases to produce the correctly sized di-tags. Biotin capture is used to remove the unwanted DNA fragments prior to the addition of a Junction Code™ Reagent. The library is re-circularized, amplified by PCR, and then sequenced on an Illumina platform.

Workflow Diagram:

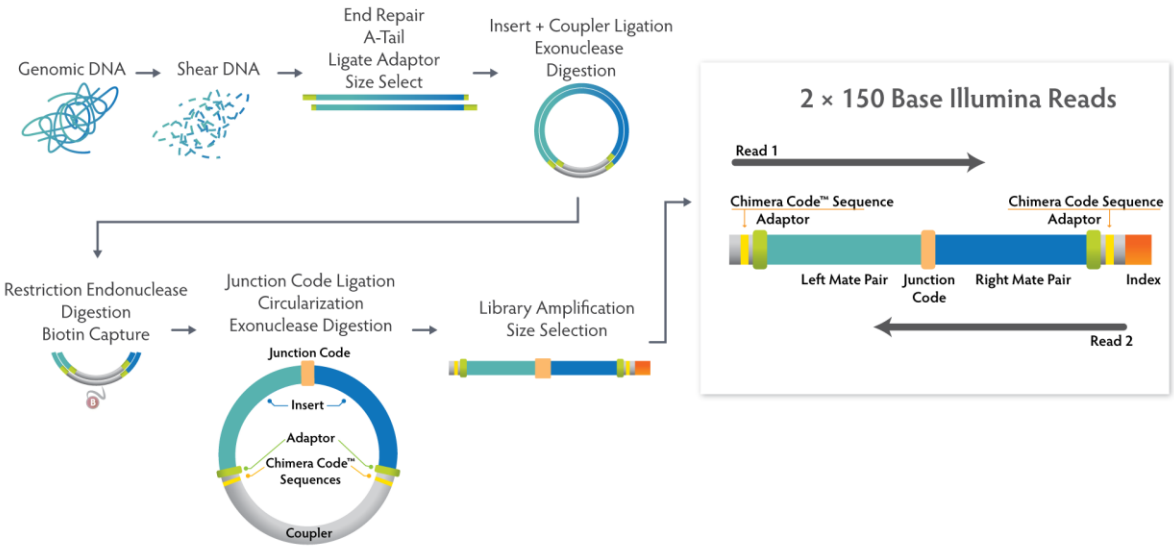


Figure 1: Schematic of NxSeq® Library construction

Workflow Timeline:

The NxSeq Long Mate Pair protocol contains multiple optional stopping points. However the protocols can be used to generate sequence ready mate pair libraries in three days. The table below lists the three-day work flow.

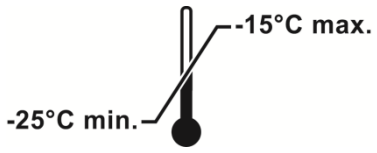
Day	Steps	Estimated Time
1	1. Shear DNA to Appropriate Size 2. Clean up of Sheared DNA 3. End Repair 4. A-Tailing 5. Ligation of Adaptor 6. Clean up of Adaptor-Ligated DNA 7. Size Selection of Adaptor- Ligated DNA 8. Ligation of Insert to Coupler (overnight ligation)	Hands On: 2 ½ hours Elapsed Time: 6 hours Note: Step 8 ends with overnight ligation.
2	9. Exonuclease Treatment 10. Clean up of Exonuclease Treated Insert / Coupler 11. Restriction Enzyme Digest 12. Biotin Capture 13. Junction Code Ligation 14. Clean up of Insert / Coupler with Junction Code 15. DNA Re-circularization 16. Exonuclease Treatment	Hands On: 2 hours Elapsed Time: 8 ½ hours
3	17. Clean up of Exonuclease Treated Coupler / Insert 18. Amplification using Accura HotStart 2X Master Mix 19. Size Selection of Amplified Mate Pair Library 20. Illumina Sequencing	Hands On: 3 hours Elapsed Time: 6 hours

Product Designations

Product	Kit Size	Catalog number	Part Number(s)
NxSeq® Long Mate Pair Library Kit	10 libraries	13000-1	A943016-1 A943018-1
NxSeq® Long Mate Pair Library and Index Kit	10 libraries + 12 indices each	13100-1	A943016-1 A943018-1 A913078-1
NxSeq® Long Mate Pair Index Kit	12 indices, 5 libraries each	13200-1	A913078-1
NxSeq® Long Mate Pair Library Kit, Box 1	10 libraries	13300-1	A943016-1
NxSeq® Long Mate Pair Library Kit, Box 2	10 libraries	13400-1	A943018-1

Components and Storage

Store all kits and components at -20 °C



NxSeq® Long Mate Pair Kit – Box 1

Reagent Name	# tubes in kit (A943016)	Cap Identifier	Map Identifier	Part Number
Elution Buffer	1	EB	EB: Elution Buffer	F882705-6
End Repair Tailing Buffer[1]	2	ERB	ERB: E.R. Buffer	F882709-6
End Repair Enzyme Mix[1]	1	ERE	ERE: E.R. Enzyme	F93034-6
Klenow Fragment	1	KF	KF: Klenow	F93626-6
Adaptor	1	ADT	ADT: Adaptor	F813105-6
Ligase	1	LIG	Lig: Ligase	F832792-6

1. Reagent only in box 1.

NxSeq® Long Mate Pair Kit – Box 2

Reagent Name	# tubes in kit (A943018)	Cap Identifier	Map Identifier	Part Number
Elution Buffer	6	EB	EB: Elution Buffer	F882705-6
Klenow Fragment	1	KF	KF: Klenow	F93626-5
Ligase	1	LIG	Lig: Ligase	F832792-5
Coupler Mix	1	CM	CM: Coupler	F823007-6
10X Ligase Buffer	1	10X	10X: Ligase Buffer	F88912-6
Nuclease 1	1	N1	N1: Nuclease 1	F832799-6
Nuclease 2	1	N2	N2: Nuclease 2	F83920-6
Biotin Wash Buffer	4	BWB	BWB: Biotin Wash	F882794-6
Biotin Capture Buffer	1	BCB	BCB: Biotin Buffer	F882711-6
Biotin Capture Reagent	1	BCR	BCR: Biotin	F812766-6

Reagent Name	# tubes in kit (A943018)	Cap Identifier	Map Identifier	Part Number
			Reagent	
Tailing Buffer	1	TB	TB: Tailing Buffer	F882713-6
Junction Code™ Reagent	1	JC	JC: Junction Code	F812816-6
T4 Polynucleotide Kinase	1	PNK	PNK	F93135-6
Accura HotStart 2X Master Mix	1	AMM	AMM: Accura 2X MM	B732793
Primer Mix, Index 12	1	12	12: Index 12	B713077

NxSeq® Long Mate Pair Index Kit

Reagent Name	# tubes in kit (A913078-1)	Cap Identifier	Map Identifier	Part Number
Primer Mix, Index 1	1	1	1: Index 1	F813066-1
Primer Mix, Index 2	1	2	2: Index 2	F813067-1
Primer Mix, Index 3	1	3	3: Index 3	F813068-1
Primer Mix, Index 4	1	4	4: Index 4	F813069-1
Primer Mix, Index 5	1	5	5: Index 5	F813070-1
Primer Mix, Index 6	1	6	6: Index 6	F813071-1
Primer Mix, Index 7	1	7	7: Index 7	F813072-1
Primer Mix, Index 8	1	8	8: Index 8	F813073-1
Primer Mix, Index 9	1	9	9: Index 9	F813074-1
Primer Mix, Index 10	1	10	10: Index 10	F813075-1
Primer Mix, Index 11	1	11	11: Index 11	F813076-1
Primer Mix, Index 12	1	12	12: Index 12	F813077-1

Customer-Supplied Reagents and Equipment

Reagent	Recommended Vendor	Catalog #
HpyCH4V Restriction Enzyme	NEB	R0620S
*Rsa*I Restriction Enzyme (10 U/µL)		R0167S
*Alu*I Restriction Enzyme (10 U/µL)		R0137S
HaeIII Restriction Enzyme (10 U/µL)		R0108S
CutSmart™ Buffer		B7204S
*Acc*II Restriction Enzyme (10 U/µL)	Takara	1002A
Dynabeads MyOne Streptavidin C1	Life Technologies	65001
Agencourt AMPure XP magnetic beads	Beckman Coulter	A63881 or A63882
100% Ethanol	Various	Various
Nuclease Free Water (not DEPC-treated)	Ambion	AM993
1.5 mL Eppendorf DNA LoBind Microcentrifuge tubes	Eppendorf	22431021
0.2 mL thin wall PCR tubes	Various	Various
Qubit® dsDNA HS Assay Kit	Invitrogen	Q32854
Bioanalyzer DNA Kits. Options include • Agilent High Sensitivity DNA Kit • Agilent DNA 12000 Kit (optional)	Agilent Technologies	5067-4626 5067-1508

Equipment	Recommended Vendor	Catalog #
Qubit Fluorometer or equivalent	Invitrogen	Q32866
Thermomixer R (16 °C – 80 °C)	Eppendorf	22670107
Heat blocks (25 °C – 80 °C)	Various	Various
Magnetic rack for 1.5-2 mL tubes (DynaMag-2 Magnet)	Invitrogen	12321D
Thermocycler with a heated lid	Various	Various
Mini-centrifuge for quick ~2000 g spins	Various	Various
2100 Bioanalyzer	Agilent	Various
Electrophoresis supplies: • Agarose • Markers (1K plus and 100 bp)	Various	Various
	Lucigen	50020-1 or 50010-1
E-gel components: • E-Gel® iBase™ and E-Gel® Safe Imager™ transilluminator combo kit • 0.8% CloneWell with SYBR® Safe • 2% E-gel® Agarose gel with SYBR® Safe	Life Technologies	G6465
		G6618-08
		G5218-02
HydroShear	Digilab Inc	See web product pages
Megaruptor	Diagenode Inc	See web product pages
Covaris G-TUBES	Covaris	520079 or 520104

Prior to starting: Restriction Enzyme Selection

Before proceeding with library construction, you must identify the restriction enzyme(s) needed to digest the gDNA to 400–900 bp (desired final library after PCR amplification). This step is critical to ensure the kit performs as designed and the sequencing coverage is uniform.

The restriction enzyme(s) needed will vary for each genome. The optimal digestion method may be multiple digests with individual restriction enzymes or multiple digests with a combination of two or more restriction enzymes. Each enzyme or combination of enzymes will produce a different digestion pattern and will add diversity to the genome coverage.

Lucigen recommends performing multiple digestion reactions using both individual restriction enzymes and combinations of enzymes provided by NEB orTakara,), and then visualize the digest results on an E-Gel®, Agilent® Bioanalyzer®, or agarose gel.

Start by digesting with the individual restriction enzymes. After visualizing these digests on a gel, subsequent combination digests can be tested. For example, two or more infrequent cutting enzymes can be combined to produce the desired digestion pattern.

See figures 2-4 below for example gels of restriction digest testing.

Restriction Enzyme Options

Enzyme	Supplier
HpyCH4V Restriction Enzyme	NEB
*Rsa*I Restriction Enzyme (10 U/µL)	
*Alu*I Restriction Enzyme (10 U/µL)	

Enzyme	Supplier
HaeIII Restriction Enzyme (10 U/µL)	
CutSmart™ Buffer	
*Acc*II Restriction Enzyme (10 U/µL)	Takara

General Digestion Set up

- Add the following reagents to 0.2 mL thin wall PCR tube(s).

Reagent	Final concentration per reaction
gDNA	200 ng
Buffer (RE or CutSmart)	2 µL
Restriction Enzyme(s)	10 Units (not to exceed 2 µL)
Nuclease-free water	To 20 µL
Total	**20 µL**

- Mix by pipetting up and down 10 times.
- Incubate reaction(s) at 37 °C for 30 min.
- Run reaction(s) as well as undigested gDNA on 1.7% gel (agarose or or E-gel) with DNA ladder (e.g. 100 bp ladder).

- Review results on gel (agarose or E-gel) (Figure 2).
- Determine optimal restriction enzyme(s).

  Ideal digests will contain the majority of the smear within a 500-700 bp size range. For optimal sequencing results, the recommendation is to perform two side by side restriction digests using at least 1 enzyme for each digest and pool the digested material prior to cleanup (see step 11 for details).

  The table below provides recommendations for restriction enzymes for four reference genomes: See *Appendix A: Example Experimental Set-up for Restriction Enzyme Testing* for an example of Restriction Enzyme testing using *E. coli.*

Genome	GC Content	Restriction Enzymes
E. coli	50%	Reaction #1: HpyCH4V Reaction #2: RsaI + HaeIII
T. aquaticus	68%	Reaction #1: AluI Reaction #2: RsaI + AccII
H. sapiens	50%	Reaction #1: AluI Reaction #2: HaeIII + RsaI

  The images below provide examples of restriction enzyme testing that were used to identify recommended restriction enzymes:

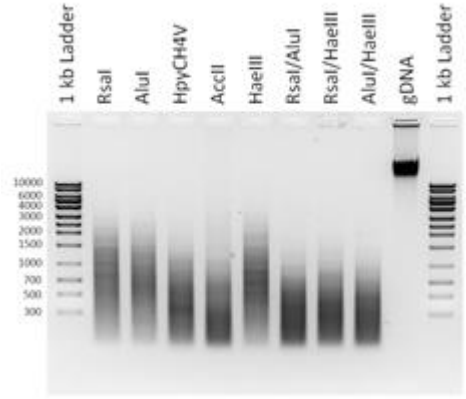


Figure 2. Results of *E. coli* restriction digest testing. Optimal results are seen with HpyCH4V and RsaI + HaeIII.

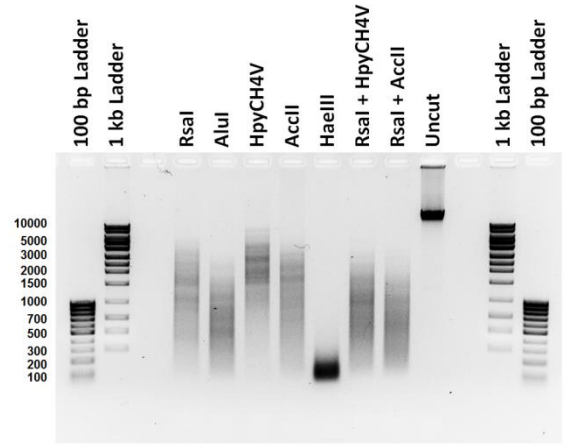


Figure 3. Results of *T. aquaticus* restriction digest testing. Optimal results are seen with AluI and RsaI + AccII

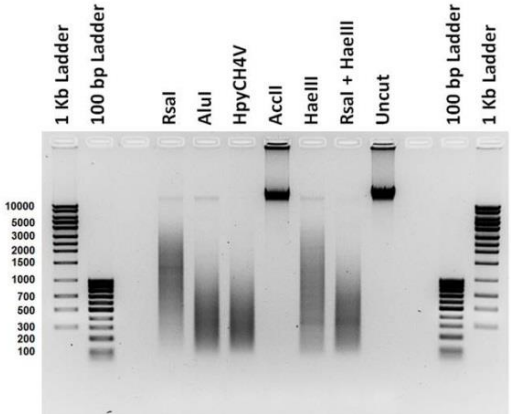


Figure 4. Results of Human gDNA restriction digest testing. Optimal results are seen with AluI and HaeIII + RsaI.

General Recommendations

- Use Eppendorf Lo-Bind 1.5 mL tubes throughout the protocol.
- Thaw all kit reagents on ice prior to use.
- Use a Qubit Fluorometer or equivalent to perform all sample quantification throughout the protocol.
    - The ratios of material used in each ligation step throughout the protocol have been optimized for the best performance.
    - The materials provided in the kit are quantified using Qubit® 2.0 Fluorometer (Life Technologies).
    - The use of other quantification methods (e.g. gel image, A260/A280), may lower the efficiency of the kit and result in insufficient material to sequence.

Detailed Protocol

1. Shear DNA to Appropriate Size

In this step, the genomic DNA (gDNA) is sheared to an average size range that is larger than the desired insert size. See *Appendix B: The effect of the size range of sheared DNA on insert size* for additional information on shearing and size selection.

Notes:

- gDNA used must be free of contaminating RNA.
- gDNA used must be of a high molecular weight (>10 kb).
- Tagmentation from Illumina should not be used for shearing, Tagmentation will add additional nucleotides, and the use of Tagmentation has not been tested with the mate pair kit.

A sample loss of 20-60% is expected during shearing and bead clean up. The percentage of sample loss will vary depending on the shearing method used. This expected loss should be taken into account when determining the amount of gDNA to shear.

Use the table below to determine the recommended amount of starting gDNA and shearing method for your final desired insert size.

Final Desired insert size	Recommended amount of starting material	Recommended shearing conditions[1]
< 2 kb	2.5-3 µg	Covaris LE220 or Diagenode Bioruptor
2 kb	2.5-3 µg	3 kb setting with the Megaruptor short hydropore
5 kb	3.5-6.3 µg	8 kb setting with the Megaruptor short hydropore 8 kb setting with Covaris G-tubes • Eppendorf® 5424 & 5415 R centrifuges: 7,200 RPM for 60 seconds • Eppendorf® MiniSpin plus: 9,400 RPM for 60 seconds
8 kb	11.5-20 µg	10 kb setting with the Megaruptor long hydropore 10 kb setting with Covaris G-tubes • Eppendorf® 5424 & 5415 R centrifuges: 6,000 RPM for 60 seconds • Eppendorf® MiniSpin plus: 8,000 RPM for 60 seconds

1. Use manufacturer's recommended protocols.

1.1 Options for shearing

- HydroShear (Digilab Inc., Marlborough, MA)
- Megaruptor (Diagenode Inc., Denville, NJ)
- Covaris G-TUBES (Covaris, Woburn, MA)

1.2 Protocol

Per manufacturer's instructions.

Notes:

- Resuspend sheared DNA in Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA) prior to proceeding to step 2.
- Sample volume after shearing may vary depending on sample type and method of shearing used.

1.3 Size confirmation of sheared gDNA

- Confirm the correct size of the sheared gDNA by visualization on a gel (agarose or 0.8% CloneWell® E-gel®).

Important Note: It is strongly recommended that agarose gel methods are used to determine the size of the insert after shearing. Size determination at this step is dependent on the method used. Modified DNA (e.g. FFPE and ChIP samples) or contaminats in the sample may result in skewed size results for chip-based methods such as Agilent BioAnalyzer.

See *Appendix B: The effect of the size range of sheared DNA on insert size*, for an example gel image.

2. Clean Up of Sheared DNA

In this step, the sheared gDNA from step 1.2 is concentrated and purified.

Important Note: Reagents used from this point through "Ligate adapters to DNA fragments" are included in box 1.

2.1 NxSeq® Long Mate Pair Kit – box 1 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

2.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Sheared gDNA	From step 1.2
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
AMPure XP Beads	Beckman Coulter
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	

2.3 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Prepare 70% Ethanol.
- Pipette the sheared gDNA from step 1.6 into 1.5 mL LoBind tube.
- In the tube with the sheared gDNA, set up the bead clean up reaction; add each reagent in the following order.

Reagent	Volume	Example
Sheared gDNA	X	150 µL (from step 1.2)
AMPure XP Beads	X	150 µL
Total	**2X**	300 µL

Note: X = volume of sheared gDNA remaining after size confirmation in step 1**.** Use a 1:1 ratio of beads to sample.

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack during incubation.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **2.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube and pipetting the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **2.3 Wash** step once more.

- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove any remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.
- With the tube in the magnetic rack, add the following reagent.

Reagent	Volume
Elution Buffer (EB)	52 µL

- Remove the tube from the magnetic rack.
- **2.3 Mix**: Mix the beads and the buffer by gently pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.

- Repeat steps **2.3 Mix** step.
- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- Remove 50 µL of sample containing purified, sheared gDNA.

2.4 Quantification of Purified, Sheared gDNA

- Quantify the sample from step 2.3 Purified, Sheared gDNA using Qubit® dsDNA HS Assay Kit with the Qubit® 2.0 Fluorometer according to manufacturer's instructions.

- Minimum amount and concentration of DNA required to proceed.

Insert Size	Minimum Amount DNA Required	Minimum Concentration DNA Required
up to 2 kb	1.0 µg	≥ 22 ng/µL (after elution)
3-5 kb	2.5 µg	≥ 50 ng/µL (after elution)
6-8 kb	8 µg	≥ 160 ng/µL (after elution)

Optional Safe Stopping Point: DNA can be stored at -20 °C.

3. End Repair

In this step, the Purified, Sheared gDNA from step 2.4 is end-repaired. Each end-repair reaction is limited by the number of DNA molecules. Therefore, the number of reactions performed at this step is determined by the insert size:

Insert Size	Recommended # of reactions
up to 2 kb	2
3-5 kb	5
6-8 kb	8

3.1 NxSeq® Long Mate Pair Kit – box 1 Reagents

Reagent	Cap Identifier
End Repair Tailing Buffer	ERB
End Repair Enzyme Mix	ERE

3.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Purified, Sheared gDNA	From step **2.4**
Nuclease Free Water	Ambion
0.2 mL thin wall PCR tubes	Eppendorf
Thermocycler	User

3.3. Protocol

- Add the following reagents to 0.2 mL thin wall PCR tubes (number of reactions determined in table in section 3: End Repair).

Reagent	Amount (for each reaction) ≤ 5kb inserts	Amount (for each reaction) > 5-8kb inserts
Purified, sheared gDNA	500 ng	1 µg
Nuclease-Free Water	Up to 23 µL	Up to 23 µL
End Repair Tailing Buffer (ERB)	25 µL	25 µL
End Repair Enzyme Mix (ERE)	2 µL	2 µL
Total	**50 µL**	**50 µL**

- Mix by pipetting up and down 10 times.
- Place tube(s) in a thermocycler and incubate according to the following parameters.

Step	Temperature	Time
1	25 °C	20 minutes
2	72 °C	25 minutes
3	4 °C	Hold

- Proceed directly to step 4: A-Tailing.

4. A-Tailing

In this step, the End-Repaired gDNA from step 3.3 is A-tailed. The number of reactions performed during this step is the same as the number of reactions performed in step 3. End Repair.

4.1 NxSeq® Long Mate Pair Kit – box 1 Reagents

Reagent	Cap Identifier
Klenow Fragment	KF

4.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
End-repaired gDNA	From step **3.3**
Thermocycler	User

4.3 Protocol

- Using the tubes containing the End-repaired gDNA, set up the A-tailing reaction; add each reagent in the following order.

Reagent	Volume (µL) (for each reaction)
End-repaired gDNA	50
Klenow Fragment (KF)	2
Total	**52**

- Mix by pipetting up and down 10 times.
- Place the tube in thermocycler and incubate according to the following parameters.

Step	Temperature	Time
1	37 °C	20 minutes
2	70 °C	15 minutes
3	4 °C	Hold

- Proceed directly to step 5. Ligation of Adaptor.

5. Ligation of Adaptor

In this step, the A-tailed gDNA from step 4.3 is ligated to the adaptor.

NOTE: Do not vortex the adaptor. Mix by pipetting up and down and spin down briefly prior to use.

5.1 NxSeq® Long Mate Pair Kit – box 1 Reagents

Reagent	Cap Identifier
Adaptor	ADT
Ligase	LIG

5.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
A-tailed gDNA	From step **4.3**
Thermocycler	User

5.3 Protocol

- In the tube with the A-tailed gDNA, set up the ligation reactions; add each reagent in the following order.

Reagent	Volume (µL) (for each reaction)
A-tailed gDNA	52
Adaptor (ADT)	6
Ligase (LIG)	4
Total	**62**

- Mix by pipetting up and down 10 times.
- Place tube in the thermocycler and incubate according to the following parameters:

Step	Temperature	Time
1	25 °C	30 minutes
2	70 °C	15 minutes
3	4 °C	Hold

- Spin the tubes briefly to collect materials at the bottom of the tubes.
- Pool all ligation reactions into one clean 1.5 mL LoBind tube.
- Calculate the total volume of pooled ligation reactions and record the value.

NOTE: This information is needed in step 6.3.

- Proceed directly to step 6. Buffer Exchange.

NOTE: After this step, there is only one tube for each sample (library).

IMPORTANT NOTE: Reagents used prior to this point are included in the box 1. Reagents used after this point are included in box 2.

6. Clean up Adaptor-Ligated DNA

In this step, the Insert with Ligated Adaptor from step 5.3 is cleaned up using a buffer exchange reaction.

6.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

6.2 Reagents / Equipment Needed

Reagent	Supplied By
Insert with Ligated Adaptor	From step **5.3**
AMPure XP Beads	Beckman Coulter
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User
1.5 mL LoBind Microcentrifuge tubes	Eppendorf

6.3 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Set up the buffer exchange reaction; add each reagent in the following order.

Reagent	**Volume per reaction (µL)**			
	1 adapter-ligated reaction	**2 pooled reactions** (up to 2 kb inserts)	**5 pooled reactions** (3-5 kb inserts)	**8 pooled reactions** (6-8 kb inserts)
Insert with Ligated Adaptor	62	124	310	496
AMPure XP Beads	62	124	310	496
Total	**124**	**248**	**620**	**992**

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **6.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube and pipetting the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **6.3 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove any remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.

- Keeping the tube in the magnetic rack, add the following reagent.

Reagent	Volume
Elution Buffer (EB)	402 µL

- Remove the tube from the magnetic rack.
- **6.3 Mix**: Mix the beads and the buffer by gently pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.

- Repeat step **6.3 Mix**.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- Remove 400 µL and transfer to a clear 1.5 mL Lo Bind tube.

Optional Safe Stopping Point: DNA can be stored at -20 °C.

7. Size Selection of Adaptor-Ligated DNA

In this step, the Cleaned Insert with Ligated Adaptor from step 6.3 is size selected.

Prior to size-selection, you must determine the required bead concentration. The appropriate bead concentration is based on the lower end of the size distribution of the desired insert length.

See *Appendix C: Determining bead concentration* for additional information.

7.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

7.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Cleaned Insert with Ligated Adaptor	From step **6.3**
AMPure XP Beads	Beckman Coulter
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User
1.5 mL LoBind Microcentrifuge tubes	Eppendorf

7.3 Determine Required Bead Concentration

The appropriate bead concentration is based on the lower end of the size distribution of the desired insert base pair length.

Low end of size distribution (bp)	Bead Concentration	Sample Volume (µL)[1]	Volume of Beads (µL)
1500	0.46	400	184
2000	0.44	400	176
2500	0.43	400	172
3000	0.42	400	168
4000	0.4	400	160
5000	0.4	400	160
8000	0.4	400	160

[1]Ensure Sample Volume is exactly 400µL. If needed add Elution Buffer (EB) to bring the volume to 400µL.

7.4 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Set up the size selection reaction; add each reagent in the following order.

Reagent	Volume (µL)	Volume (µL) for 5kb Insert
Cleaned Insert with Ligated Adaptor	400[1]	400[1]
AMPure XP Beads Volume determined above	X (see table above)	160
Total	**400 + X**	**560**

[1]Ensure Sample Volume is exactly 400µL. If needed, add Elution Buffer (EB) to bring the volume to 400µL.

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack during the incubation.

- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **7.4 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube and pipetting the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **7.4 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove all remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.
- Keeping the tube in the magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	52

- Remove the tube from the magnetic rack.
- **7.4 Mix**: Mix the beads and the buffer by gently pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.

- Repeat step **7.4 Mix**.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- Remove 50 µL and transfer to a clean 1.5 mL LoBind tube.

7.5 Concentration: Quantify using Qubit according to manufacturer's instructions.

- Record the concentration in ng/µL.

  NOTE: This information is "Z" in step 8.3

- Minimum amount and concentration of DNA required to proceed.

Insert Size	Minimum Amount DNA Required	Minimum Concentration DNA Required
up to 2 kb	100 ng	2.0 ng/µL
3-5 kb	250 ng	5.0 ng/µL
6-8 kb	400 ng	8.0 ng/µL

7.6 Size: Confirm the correct size selection by one of the following methods:

- Visualization on gel (agarose or E-gel).
- Bioanalyzer trace using Agilent DNA 12000 kit.

  See *Appendix B: The effect of the size range of sheared DNA on insert size*, for an example gel image.

8. Ligation of Insert to Coupler

In this step, the size-selected DNA with adaptor from step 7.4 is ligated to the coupler.

8.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Coupler mix	CM
10X Ligase Buffer	10X
Ligase (LIG)	LIG

8.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Size-selected DNA with Adaptor	From step 7.4.
Nuclease Free Water	Ambion
Thermomixer (set at 16 °C)	Eppendorf
Thermomixer or heat block (set at 70 °C)	Eppendorf
Pipet designed for volumes under 2µL	Various

8.3 Determine Amount of Insert Required

- Use the following equation to determine the amount of size-selected DNA with adaptor material required for step 8.4 Protocol.

NOTE: The optimal condition for this step is to use equal amounts of insert and coupler in the ligation reaction. The size of the coupler (included in the kit) is 2000 bp and the amount of coupler specified for each reaction is 100 ng.

$$\frac{\boldsymbol{insert\ size\ bp}}{\boldsymbol{2000\ bp}}\boldsymbol{x\ 100\ ng = ng\ insert = Y}$$

Example: $\frac{\boldsymbol{5000\ bp}}{\boldsymbol{2000\ bp}}\boldsymbol{x\ 100\ ng = \ 250\ ng}$

8.3.1 Calculate and record the amount of insert required.

- Use the following equation to determine the required volume of size-selected DNA with adaptor required (X), based on the concentration determined in 7.4 Concentration (Z) and the amount, in ng, of insert (Y) calculated above.

$$\frac{\boldsymbol{Y\ (Amount\ in\ ng)}}{\boldsymbol{Z\ (Concentration\ in\ \frac{ng}{uL})}} = \boldsymbol{X\ volume\ in\ uL}$$

8.3.2 Calculate and record the volume of insert required.

8.4 Protocol

- In a fresh, LoBind tube, set up the ligation reaction; add each reagent in the following order.

Reagent	Volume (µL)
Size-selected Insert with adaptor	X (calculated in step 8.3.2)
Coupler mix (CM)	3
Nuclease-free water	Up to 356.5
10X Ligase Buffer (10X)	40
Ligase (LIG)	0.5
Total	**400**

 **NOTE**: Use pipet designed for volumes under 2 µL to pipet Ligase.

- Mix gently by pipetting up and down 10 times.
- Place the tube in a thermomixer.

- Incubate and heat kill the Ligated Insert/Coupler from step 8.4 according to the table below:

Step	Temperature	Time
1	16ºC	Overnight (14-16 hours)
2	70ºC	15 minutes

- Place the tube on ice for 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.
- Proceed directly to Step 9: Exonuclease Treatment.

9. Exonuclease Treatment

In this step the heat killed ligated Insert/Coupler from step 8.4 is treated to remove any linear DNA.

9.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Nuclease 1	N1
Nuclease 2	N2

9.2 Reagents / Equipment Needed

Reagent	Supplied By
Heat Killed Ligated Insert/Coupler	From Step 8.4
Thermomixer or heat block (set at 37°C)	Eppendorf
Thermomixer or heat block (set at 80°C)	Eppendorf

9.3 Protocol

- In the tube with the Heat Killed Ligated Insert/Coupler, set up the exonuclease treatment; add each reagent in the following order.

Reagent	Volume (µL)
Heat Killed Ligated Insert/Coupler	400
Nuclease 1 (N1)	7
Nuclease 2 (N2)	5
Total	**412**

- Mix gently by pipetting up and down 10 times.
- Place tube in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	37 °C	30 minutes
2	80 °C	30 minutes

- Place the tube on ice for 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.
- Proceed directly to Step 10: Clean up of Exonuclease Treated Insert/Coupler.

10. Clean Up of Exonuclease Treated Insert / Coupler

In this step, the Exonuclease Treated Insert/Coupler from step 9.3 is cleaned.

10.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

10.2 Reagents / Equipment Needed

Reagent	Supplied By
Exonuclease Treated Insert/Coupler	From step 9.3.
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
AMPure XP Beads	Beckman Coulter
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User

10.3 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Measure volume of Exonuclease Treated Insert/Coupler from step 9.3.
- In the tube of the Exonuclease Treated Insert/Coupler, set up the bead clean up reaction; add each component in the following order.

Reagent	Volume (µL)
Exonuclease Treated Insert/Coupler	412[1]
AMPure XP Beads	412[1]

[1]Use equal volumes of Exonuclease Treated Insert/Coupler and AMPure beads.

- Perform all of the following steps at room temperature.
- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **10.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube and pipetting the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **10.3 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove any remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.
- With the tube in the magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	37

- Remove the tube from the magnetic rack.

- **10.3 Mix**: Mix the beads and the buffer gently by pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.

- Repeat step **10.3 Mix.**
- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- Transfer 17 µL each into two clean 1.5 mL LoBind tubes.

Optional Safe Stopping Point: DNA can be stored at -20 °C.

11. Restriction Enzyme Digest

In this step, the cleaned insert/coupler from step 10.3 is digested with multiple restriction enzymes. The choice of enzyme(s) will determine the library size for sequencing (determined in Pre-Work, *Restriction Enzyme Selection).*

Note: Random shearing methods, such as Gtubes or Megaruptor, must not be used in place of restriction enzyme digests.

11.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

None

11.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Cleaned Insert/Coupler	From step 10.3
Restriction Enzyme(s)	NEB or Takara
CutSmart Buffer	NEB
Thermomixer or heat block (set at 37 °C)	Eppendorf
Thermomixer or heat block (set at 80 °C)	Eppendorf

11.3 Protocol

- To each tube from step 10.3, set up the restriction enzyme digests adding each reagent in the following order. For optimal sequencing results, we recommend using different restriction enzymes or enzyme pools for Rxn 1 and Rxn 2 (see Prior to starting: Restriction Enzyme Selection, page 7).

Reagent	Rxn 1 Volume (µL)	Rxn 2 Volume (µL)
Cleaned, Insert/Coupler	17	17
CutSmart Buffer [1]	2	2
Restriction Enzyme or Restriction Enzyme pool[2]	1	1
Total	**20**	**20**

[1] Use of CutSmart Buffer (NEB) is highly recommended. Buffers from other vendors have not been tested and may not be compatible with the restriction enzyme(s) used to digest the gDNA.
[2] If using a pool of restriction enzymes, mix 10 Units of each enzyme in a 1.5 mL LoBind tube and use 1 µL for the digest.

- Mix gently by pipetting up and down 10 times.
- Place tubes in a thermomixer or heat block and incubate according to the instructions below.

Step	Temperature	Time
1	37 °C	30 minutes
2	80 °C	15 minutes

- Place the tube on ice for at least 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.
- Combine restriction digested material into one 1.5 mL LoBind tube.
- Keep tube on ice until next step to prevent digestion by inactivated restriction enzyme.
- Proceed directly to Step 12: Biotin Capture.

12. Biotin Capture

In this step, the target sequence (insert / coupler) from step 11.3 will be captured and purified to remove competing fragments.

12.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Biotin Wash Buffer	BWB
Biotin Capture Buffer	BCB
Biotin Capture Reagent	BCR
Elution Buffer	EB

12.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Streptavidin beads (MyOne C1)	Invitrogen
Nuclease Free Water	Ambion
Digested Insert/Coupler	From step **11.3**
Thermomixer or heat block (set at 50 °C)	Eppendorf
Thermomixer or heat block (set at 25 °C)	Eppendorf
Thermomixer or heat block (set at 65 °C)	Eppendorf

12.3 Prepare Streptavidin Beads (MyOne C1)

- Equilibrate Elution Buffer (EB), Biotin Wash Buffer (BWB), and Biotin Capture Buffer (BCB) to room temperature prior to use.
- Add the following reagents to a clean 1.5 mL LoBind tube.

Reagent	Volume (µL)
Streptavidin Beads (MyOne C1)	40
Biotin Wash Buffer (BWB)	100
Total	**140**

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- Remove the tube from the magnetic rack.
- Add 100 uL of Biotin Wash Buffer (BWB).
- Mix gently by pipetting up and down 10 times.

- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.
- With the tube in the magnetic rack and add the following reagent.

Reagent	Volume (µL)
Biotin Wash Buffer (BWB)	10

- Remove the tube from the magnetic rack.
- Mix gently by pipetting up and down 5 times. (Do not vortex.)
- Keep beads on ice until ready for use.

12.4 Protocol

- To the tube containing the digested insert/coupler from step 11.3, set up the Streptavidin bead clean up reaction; add each component in the following order.

Reagent	Volume (µL)
Digested Insert/Coupler from step 11.3	40
Biotin Capture Buffer (BCB)	45
Nuclease Free Water	4
Biotin Capture Reagent (BCR)	1
Total	**90**

- Mix gently by pipetting up and down 10 times.
- Place tube in thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	50 °C	30 minutes
2	25 °C	15 minutes

- Spin the tube briefly to collect materials at the bottom of the tube.

- Add the following reagent to the tube.

Reagent	Volume (µL)
Washed Streptavidin Beads from step 12.3	10

- Unless instructed otherwise, perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Place tube in thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	25 °C	15 minutes

- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **12.4 Wash**: With the tube in a magnetic rack, add 100 µL of Biotin Wash Buffer (BWB). Remove the tube from the magnetic rack and re-suspend thoroughly by gently pipetting up and down 10 times. Place the tube in magnetic rack until the supernatant becomes clear (~5 minutes). Remove the supernatant using a pipette and discard.

- Repeat the **12.4 Wash** step TWICE more, for a total of three washes.

- With the tube in the magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	52

- Remove the tube from the magnetic rack.
- Mix the beads and the buffer by gently pipetting up and down 10 times. (Do not vortex.)
- Place tube in thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	65 °C	10 minutes

- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- Remove 50 µL and place in a clean 1.5 mL LoBind tube.
- Proceed directly to Step 13: Junction Code Ligation.

13. Junction Code Ligation

In this step, the Junction Code will be ligated to the Biotin Captured Insert/Coupler from step 12.4.

Note: Before you begin, bring the Tailing Buffer to room temperature. A precipitate will form upon thawing; vortex to bring Tailing Buffer back into solution prior to use.

13.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Tailing Buffer	TB
Klenow Fragment	KF
Junction Code Reagent	JC
Ligase	LIG

13.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Biotin Captured Insert/Coupler	From step 12.4
Thermomixer or heat block (set at 25 °C)	Eppendorf
Thermomixer or heat block (set at 37 °C)	Eppendorf
Thermomixer or heat block (set at 70 °C)	Eppendorf

13.3 Protocol

- To the tube containing the biotin captured insert/coupler from step 12.4, set up the ligation reaction; add each reagent in the following order.

Reagent	Volume (µL)
Biotin Captured Insert/Coupler from step 12.4	50
Tailing Buffer (TB)	50
Klenow Fragment (KF)	6
Total	**106**

- Mix gently by pipetting up and down 10 times.
- Place the tube in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	37 °C	30 minutes
2	70 °C	15 minutes

- Place the tube on ice for 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.
- Add the following reagents.

Reagent	Volume (µL)
Biotin Captured DNA from step **13.3**	106
Junction Code Reagent (JC)	6
Ligase (LIG)	8
Total	**120**

- Mix gently by pipetting up and down 10 times.
- Place the tube in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	25 °C	30 minutes
2	70 °C	15 minutes

- Place the tube on ice for 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.
- Proceed directly to Step 14: Clean Up of Insert / Coupler with Junction Code.

14. Clean Up of Insert / Coupler with Junction Code

In this step, the Insert/Coupler Ligated with Junction Code from step 13.3 is cleaned.

14.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

14.2 Reagents / Equipment Needed

Reagent	Supplied By
Insert/Coupler Ligated with Junction Code	From step 13.3.
AMPure XP Beads	Beckman Coulter
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User

14.3 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Set up the bead clean up; add each reagent in the following order.

Reagent	Volume (µL)
Insert/Coupler Ligated with Junction Code	120
Elution Buffer (EB)	30
AMPure XP Beads	150
Total	**300**

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack.
- Place tube in magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **14.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube and pipetting the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **14.3 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove all remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.

- With the tube in the magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	52

- Remove the tube from the magnetic rack.

- **14.3 Mix**: Mix the beads and the buffer gently by pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.

- Repeat the **14.3 Mix** step.

- Place the tube in a magnetic rack until the supernatant becomes clear (~2minutes).
- Remove 50 µL and place in a clean 1.5 mL LoBind tube.

Optional Safe Stopping Point: DNA can be stored at -20 °C.

15. DNA Re-circularization

In this step, the Cleaned Ligated Insert/Couplers are re-circularized.

15.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
10X Ligase Buffer	10X
Ligase	LIG
T4 Polynucleotide Kinase	PNK

15.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Cleaned Ligated Insert/Coupler	From step 14.3
Nuclease Free Water	Ambion
Thermomixer or heat block (set at 25 °C)	Eppendorf
Thermomixer or heat block (set at 70 °C)	Eppendorf

15.3 Protocol

- Set up the ligation reaction; add each reagent in the following order.

Reagent	Volume (µL)
Cleaned Ligated Insert/Coupler	50
Nuclease Free Water	126
10X Ligase Buffer (10X)	20
T4 Polynucleotide Kinase (PNK)	2
Total	**198**

- Mix gently by pipetting up and down 10 times.
- Place the tube in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	25 °C	10 minutes

- Add the following to the reaction:

Reagent	Volume (µL)
Kinased Reaction	198
Ligase (LIG)	2
Total	**200**

Mix gently by pipetting up and down 10 times.

- Place the tube in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	25 °C	45 minutes
2	70 °C	15 minutes

- Place the tube on ice for 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.
- Proceed directly to step 16: Exonuclease Treatment.

16. Exonuclease Treatment

In this step, the re-circularized Insert/Coupler from step 15.3 is treated to remove linear DNA.

16.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Nuclease 1	N1
Nuclease 2	N2

16.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Re-circularized Insert/Coupler	From step 15.3
Thermomixer or heat block (set at 37 °C)	Eppendorf
Thermomixer or heat block (set at 80 °C)	Eppendorf

16.3 Protocol

- Set up the exonuclease reaction; add each reagent in the following order.

Reagent	Volume (µL)
Re-circularized Insert/Coupler	200
Nuclease 1 (N1)	3
Nuclease 2 (N2)	2.5
Total	**205.5**

- Mix gently by pipetting up and down 10 times.
- Place the tube in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	37 °C	30 minutes
2	80 °C	30 minutes

- Place the tube on ice for 2 minutes.
- Spin the tube briefly to collect materials at the bottom of the tube.

Safe Stopping Point: DNA can be stored at -20 °C.

17. Clean Up of Exonuclease Treated Insert / Coupler

In this step, the Exonuclease Treated Insert/Coupler from step 16.3 is cleaned.

17.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

17.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Exonuclease Treated Insert/Coupler	From step 16.3
AMPure XP Beads	Beckman Coulter
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User

17.3 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.

- Set up the bead clean up reaction; add each reagent in the following order.

Reagent	Volume (µL)
Exonuclease Treated Insert/Coupler	205.5
AMPure XP Beads	205.5

- Perform all of the following steps at room temperature.
- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack during the incubation.
- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **17.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube and pipetting the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **17.3 Wash** steps.
- Remove all remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.

- With the tube in a magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	22

- Remove the tube from the magnetic rack.

- **17.3 Mix**: Mix the beads and the buffer gently by pipetting up and down 10 times. (Do not vortex.)

- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.

- Repeat the **17.3 Mix** step.
- Place the tube in a magnetic rack until the supernatant becomes clear (~2 minutes).
- Remove 20 µL and place in a clean 1.5 mL LoBind tube.

Optional Safe Stopping Point: DNA can be stored at -20 °C.

18. Amplification Using Accura HotStart 2X Master Mix

In this step, the exonuclease-treated insert/coupler from step 17.3 is amplified.

Prior to full amplification of the library, we recommend that you perform a test reaction to determine the optimal number of cycles to reduce the potential for over-amplification. If you see inconsistent performance (e.g. amplification artifacts or over-amplification) using 2 µL of template and 20 cycles, additional test reactions may be necessary. See *Appendix D Additional Information on Amplification Artifacts* for more information.

After determining the optimal number of cycles for amplification (using instructions below), generate a fully amplified library for subsequent size selection and sequencing.

18.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB
Accura HotStart 2X Master Mix	AMM
Primer Mix - Index 12	12
NxSeq® Long Mate Pair Library Index Kit	1-12

18.2 Reagents / Equipment Needed

Reagent	Supplied By
Cleaned Treated Insert/Coupler	From step 17.3
Nuclease Free Water	Ambion
AMPure XP Beads	Beckman Coulter
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
Magnetic rack for 1.5-2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User
Thermocycler	User

18.3 Identify Optimal Number of Cycles (Test Amplification)

IMPORTANT NOTE: For multiplexing, set up one reaction per Index. Use a different Index for each library. See Appendix E for selection of compatible Index Primers.

- Set up PCR reaction (volume specified in µL).

Reagent	Volume (µL)
Accura HotStart 2X Master Mix (AMM)	10
Primer Mix - Index 12 (Cap Identifier: 12)[1]	2
Nuclease-free water	6
Mate Pair Library DNA (Step 17.3)	2
Total	**20**

[1]If multiplexing, use an appropriate Primer Mix - Index for each library. (See Appendix E.)

- **18.3 PCR Reaction**: Place the tube in a thermocycler and cycle according to the following parameters.

Step	Temperature	Time
1	94 °C	2 minutes
2	94 °C	15 seconds
3	60 °C	15 seconds
4	72 °C	60 seconds
5	Repeat steps 2-4 for 19 more cycles	
6	72 °C	5 minutes
7	4 °C	Hold

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Purify the amplified reaction: Transfer the contents of the tube(s) from step 18.3 PCR Reaction (20 µL each) to a 1.5 mL LoBind tube and add the following reagents.

Reagent	Volume (µL)
18.3 PCR Reaction from step 18.3	20
Elution Buffer (EB)	30
AMPure XP Beads	90
Total	**140**

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack during incubation.
- Place the tubes in a magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **18.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube; pipet the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **18.3 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove all remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.
- With the tubes in a magnetic rack, add the following reagent.

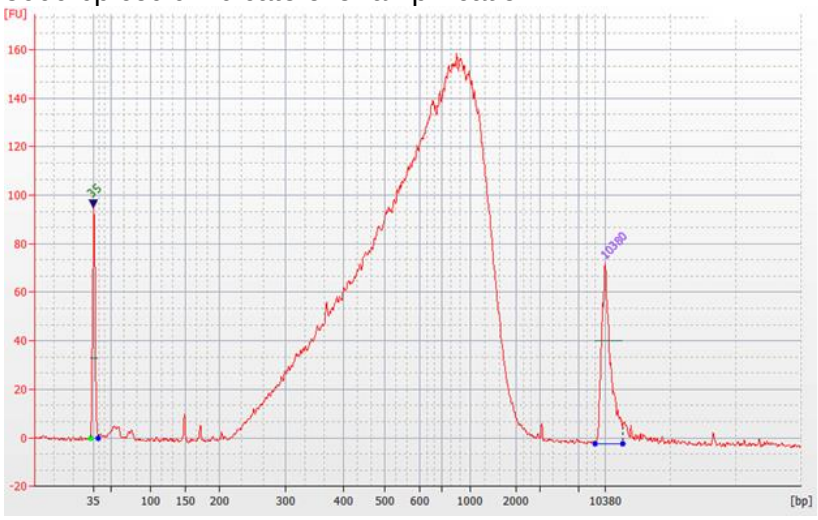
Reagent	Volume (µL)
Elution Buffer (EB)	22

- Remove the tube from the magnetic rack.
- Mix the beads and the buffer by pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation. Mix the beads and buffer by pipetting up and down 10 times.
- Place the tubes in a magnetic rack until the supernatant becomes clear (~2 minutes).

- Remove 20 µL and place in a clean 1.5 mL LoBind tube.

18.4 Size Confirmation Analyze 1 µL of the purified PCR amplification on Bioanalyzer® High Sensitivity Chip and compare to example traces below.

Ideal traces will show a peak in size distribution between ~150 - 2000 bp. Product larger than 5000 bp could indicate over amplification.



Example trace of amplified library using Agencourt® AMPure® XP Reagent.

18.5 Concentration: Quantify 1-5 µL of test amplification using Qubit® dsDNA HS Assay Kit with the Qubit® 2.0 Fluorometeraccording to manufacturer's instructions.

- Use the concentration determined in step 18.5 and the table below to identify the number of PCR Cycles Needed for Optimal Library Amplification.

  The minimum recommended concentration of amplified product is 0.3 ng/µL, or 6 ng total in 20 µL of bead-purified PCR product. After quantifiying the Test PCR (20 cycles with 2 µL library), refer to this table to identify the number of cycles that exceeds this minimum threshold while avoiding over-amplification. Determine the amount of Final Library needed for your application (Pre-Size Selection) and select the number of PCR cycles required for the 8-reaction Bulk PCR. If necessary, perform a second Test amplification to confirm your cycle number selection.

  Green indicates an optimal amount of DNA for size selection and sequencing. Yellow indicates slightly lower or slightly higher amounts of DNA; estimates within these values may be used. Red indicates either an insufficient amount of DNA for sequencing or potential overamplification of library; estimates within these values should not be used.

  If test PCR yields more than 63.76 ng, fewer than 17 cycles may be used in the 8-reaction bulk PCR.

*Test PCR:	**Estimated Final Library (Pre-Size Selection)								
20 Cycles	15 Cycles	16 Cycles	17 Cycles	18 Cycles	19 Cycles	20 Cycles	21 Cycles	22 Cycles	23 Cycles
1.04 ng						8.3 ng	15.0 ng	27.0 ng	48.6 ng
1.88 ng						15.0 ng	27.0 ng	48.6 ng	87.5 ng
3.36 ng						27.0 ng	48.6 ng	87.5 ng	157.5 ng
6.08 ng						48.6 ng	87.5 ng	157.5 ng	283.4 ng
10.94 ng					48.6 ng	87.5 ng	157.5 ng	283.4 ng	510.1 ng
19.68 ng				48.6 ng	87.5 ng	157.5 ng	283.4 ng	510.1 ng	
35.40 ng			48.6 ng	87.5 ng	157.5 ng	283.4 ng	510.1 ng		
63.76 ng		48.6 ng	87.5 ng	157.5 ng	283.4 ng	510.1 ng			
114.78 ng	48.6 ng	87.5 ng	157.5 ng	283.4 ng	510.1 ng				
≥ 206.60 ng	87.5 ng	157.5 ng	283.4 ng	510.1 ng					

* (quantified PCR product in ng/µL) x (20 µL) = Test PCR at 20 cycles
** (quantified PCR product in ng/µL) x (20 µL) x (8 reactions) = Estimated Final Library

Notes:

- The table above was generated using *E. coli* (50% GC content). The efficiency of the PCR reactions and therefore the applicability of the table above, will depend on the GC content of the genome.
- Fewer cycles will reduce the potential for over-amplification.
- Amplifying more template will increase the complexity of your library. Amplifying the entire library will provide the greatest complexity.

IMPORTANT NOTE: Amplification of genomes with very high (> 60%) or low (< 40%) GC content may result in low yield. If the Test PCR reaction (step 18.3) shows minimal or no amplification, it is recommended to substitute a polymerase that has been optimized for PCR of extreme genomes. However, these polymerases may result in lower fidelity and could affect sequence accuracy. Examples of alternative polymerases for amplifying high/low GC content genomes include:

- KAPA Biosciences Library Amplification Kit (catalog # KK2611)
- Phusion® Hot Start Flex 2X Master Mix (catalog # M0536S)
- Takara Ex Taq® DNA polymerase, HotStart version (catalog #RR006A)

18.6 Library Amplification for Size Selection and Sequencing

- Generate Master Mix for 8 reactions per library according to the table below.

IMPORTANT NOTE: For multiplexed libraries, each library will be amplified using a Master Mix containing a unique Primer Mix – Index. After amplification and bead clean up (step 19), the libraries will need to be quantified individually and pooled prior to sequencing. See *Appendix E: Additional Instructions forSample Pooling Prior to Sequencing.*

Reagent	*Volume (µL) per rxn	Master Mix Volume (µL)
Accura HotStart 2X Master Mix (AMM)	10	80
Primer Mix - Index 12 (Cap Identifer 12) [1]	2	16
Nuclease-free water	6	48
Mate Pair Library DNA (Step 17.3)	2	16
Total	**20**	**160**

[1]If multiplexing, use an appropriate Primer Mix – Index for each library.

- Aliquot 20µL of Master Mix into each 0.2-mL thermocycler tube.

- Place the tubes in a thermocycler and cycle according to the following parameters.

Step	Temperature	Time
1	94 °C	2 minutes
2	94 °C	15 seconds
3	60 °C	15 seconds
4	72 °C	60 seconds
5	Repeat steps 2-4 for X more cycles[1]	
6	72 °C	5 minutes
7	4 °C	Hold

[1] Number of PCR cycles determined in step 18.3

- **18.6 Pooled Reaction**: Pool all PCR reactions into a clean 1.5 mL LoBind tube.

18.7 Purify amplified reactions

- Add AMPure XP beads to the DNA.

Reagent	Volume (µL)
18.4 Pooled Reaction	160
AMPure XP Beads	288

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack during the incubation.
- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **18.7 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube; pipet the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **18.7 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.
- Remove any remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.
- With the tube in a magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	202

- Remove the tube from the magnetic rack.
- Mix the beads and the buffer by pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation.
- Mix the beads and buffer by pipetting up and down 10 times.
- Place the tube in a magnetic rack until the supernatant becomes clear (~2 minutes).
- Remove 200 µL and place in a clean 1.5 mL LoBind tube.

Optional Safe Stopping Point: DNA can be stored at -20 °C.

19. Size Selection of Amplified Mate Pair Library

In this step, the purified, amplified Mate Pair Library from step 18 is size selected.

IMPORTANT NOTE: For multiplexed libraries, libraries that will be sequenced in one sequencing run will need to be pooled after size selection and final quantification. See *Appendix E: Additional Instructions for Sample Pooling Prior to Sequencing* for instructions on sample pooling.

19.1 NxSeq® Long Mate Pair Kit – box 2 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

19.2 Reagents / Equipment Needed

Reagent	Supplied By
Mate Pair Library	From step 18
AMPure XP Beads	Beckman Coulter
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
Magnetic rack for 1.5 - 2 mL tube	Invitrogen
70% Ethanol (Prepare fresh daily)	User

19.3 Protocol

- Equilibrate AMPure beads to room temperature for at least 30 minutes. Vortex the beads to resuspend them.
- Equilibrate Elution Buffer (EB) to room temperature prior to use.
- Set up the bead size selection reaction; add the beads to the Mate Pair Library.

Reagent	Volume (µL) ≥ 300 bp cutoff
Mate Pair Library	200
AMPure XP Beads	130

- Perform all of the following steps at room temperature.

- Mix gently by pipetting up and down 10 times.
- Incubate at room temperature for 5 minutes; do not use a magnetic rack during incubation.

- Place the tube in a magnetic rack until the supernatant becomes clear (~5 minutes).
- With tube in the magnetic rack, remove the supernatant with a pipette and discard.

- **19.3 Wash**: Wash the beads by adding 750 µL of 70% ethanol to the tube; pipet the ethanol up and down 4 times without disturbing the beads. Remove the ethanol by pipetting and discard.

- Repeat the **19.3 Wash** step.
- Spin briefly to collect material in the bottom of the tube and place the tube back into the magnetic rack.

- Remove all remaining ethanol and let the bead pellet air dry for approximately 5 minutes while still in the magnetic rack.

- With the tube in a magnetic rack, add the following reagent.

Reagent	Volume (µL)
Elution Buffer (EB)	22

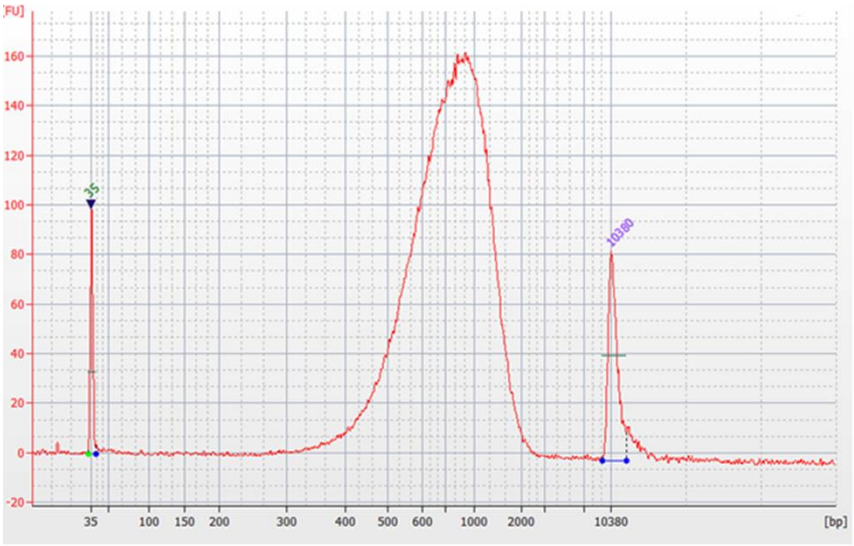
- Remove the tube from the magnetic rack.
- Mix the beads and the buffer by pipetting up and down 10 times. (Do not vortex.)
- Incubate at 37 °C for 5 minutes; do not use a magnetic rack during the incubation. Mix the beads and buffer by pipetting up and down 10 times.
- Place the tube in a magnetic rack until the supernatant becomes clear (~2 minutes).
- Remove 20 µL and place in clean 1.5 mL LoBind tube.

19.4 Concentration: Quantify using Qubit® dsDNA HS Assay and Qubit® 2.0 Fluorometer according to manufacturer's instructions.

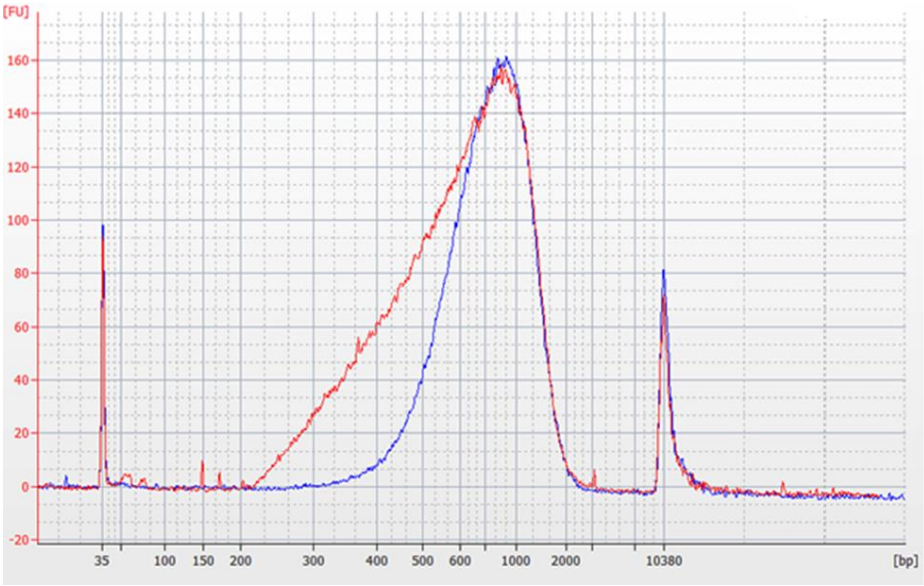
- Ensure that your sample is the appropriate concentration for your sequencing system or provider.

19.5 Size Confirmation Cleaned Mate Pair Library

- Dilute 1 µL of library to ~1 ng/µL. Analyze the diluted library on the Bioanalyzer® High Sensitivity Chip to determine size distribution.



Example trace of final size selected library using Agencourt® AMPure®XP Reagent.



Example trace of overlaid samples: amplified library vs. size selected library.

Optional Safe Stopping Point: DNA can be stored at -20 °C.

20. Illumina Sequencing

- You must spike a 5% PhiX Control (Illumina) into the mate pair library sample or sample pool prior to loading on sequencer.

  Important Note: The PhiX control is critical to ensure sufficient diversity of the first 30 cycles of the sequencing run. The information generated from the PhiX control can also be used to confirm the sequencer and sequencing reagents are performing as expected. If performance issues are seen and the PhiX control was not spiked into the sample, Lucigen will not be able to eliminate the sequencing as the cause of the issues, and therefore will not replace any NxSeq Long Mate Pair Library kits.

  Information on the expected performance of the PhiX control can be found on Illumina's website:
  - MiSeq: http://www.illumina.com/systems/miseq/performance_specifications.ilmn
  - HiSeq2500: http://www.illumina.com/systems/hiseq_2500_1500/performance_specifications.ilmn

- Proceed with Illumina sequencing with the MiSeq, NexSeq 500, or the HiSeq2500 using 300, 500, or 600 cycle chemistries. The mate pair libraries should not be sequenced using 50, 75, and 150 cycle chemistries for any of the three Illumina Platforms. These kits do not have enough cycles to read through the Chimera Code and Insert Adaptor portions of the library.
- If multiplexing, normalize and pool samples according to the appropriate Illumina Platform User's Manual.

Note: The Illumina SAV and BaseSpace Data By Cycle charts generated for NxSeq® Mate Pair libraries will look different from SAV charts generated for Nextera libraries. A non-random variable length spacer was added between the sequencing primer binding site and the chimera code sequences to shift downstream sequence and prevent sequencing software errors. This results in variable intensity for the first 30 cycles of Read 1 and Read 2 of a sequencing run. See figures below for example charts.

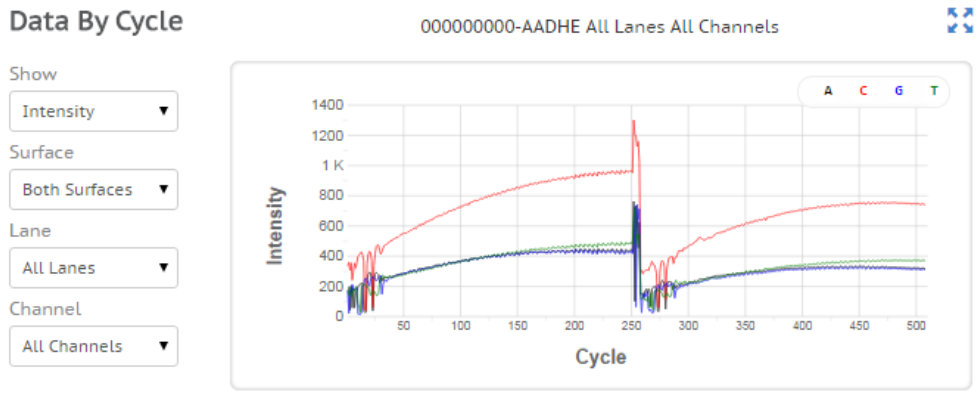


Figure 1: NxSeq Long Mate Pair K12 *E. coli* Libraries sequenced on the MiSeq using the MiSeq Reagent Kit v2 (500 cycle).

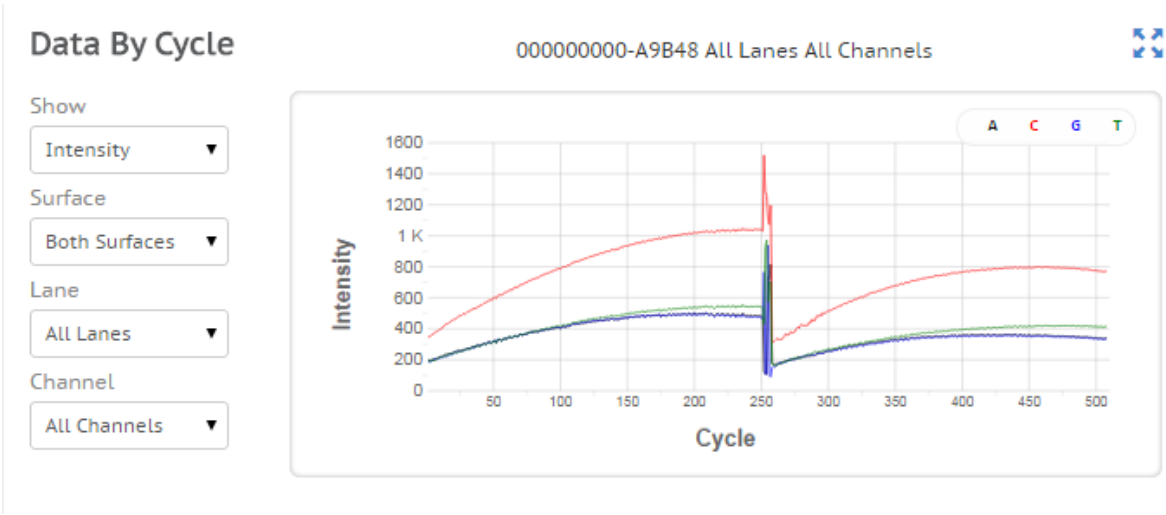


Figure 2: Nextera Mate Pair K12 *E. coli* Libraries sequenced on the MiSeq using the MiSeq Reagent Kit v2 (500 cycle).

21. Analysis of Sequencing Data from Illumina Instruments

The raw sequencing reads from Illumina instruments contain a variety of Chimera codes, adaptor codes, and Junction Code combinations. It is necessary to filter and trim the raw reads prior to assembly according to the following workflow, using scripts written in Python. The scripts can be found at: http://www.lucigen.com/NGS-Long-Read-Mate-Pair-Scripts-Sample.html. After this filtering, the data can be assembled using open source or commercially available software.

Alternatively, the open source assembler SPAdes 3.5 is capable of using raw sequencing reads during assembly, performing all filtering, trimming, and mate pair splitting internally.

See *Appendix G: Sequence Analysis* for details on the filtering process, scripts, and assembly software options.

See *Appendix H: Sequence and Location Information* for information on the sequences and location of Adaptor, Chimera Code™ Sequences, and Junction Code™ Sequences.

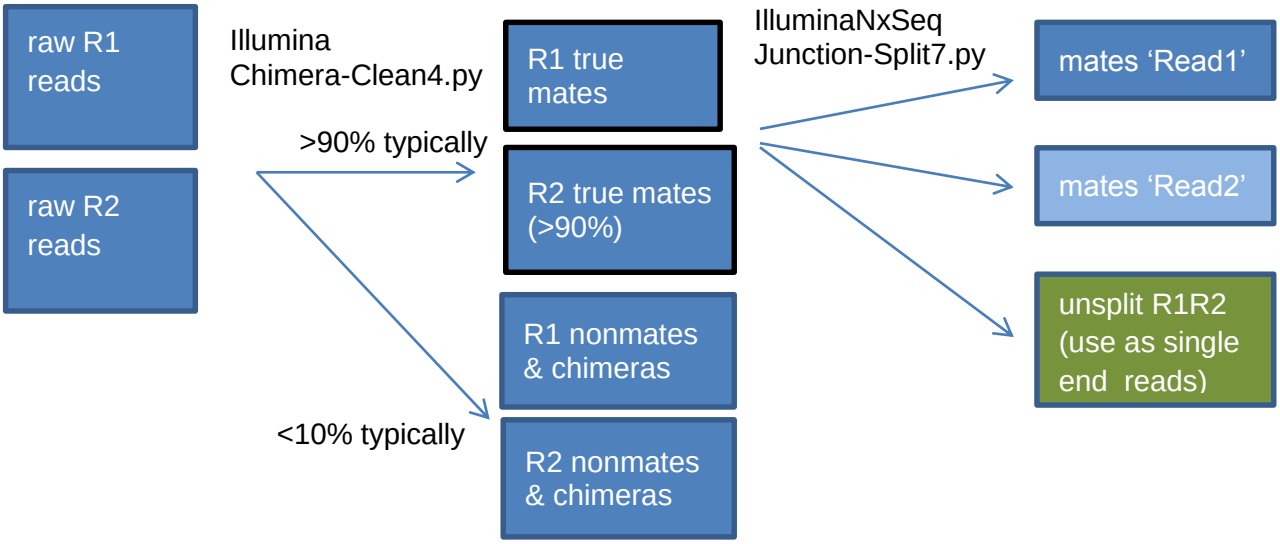


Figure 1: Workflow to filter and trim the raw reads prior to assembly.

Appendix A. Example Experimental Set-up for Restriction Enzyme Testing

The table below provides an example experimental set up to identify the appropriate restriction enzymes for *E. coli.* Note the use of individual restriction enzymes and pooled restriction enzymes.

- Add the following reagents to 0.2 mL thin wall PCR tubes.

Reagent	**1 (µL)**	**2 (µL)**	**3 (µL)**	**4 (µL)**	**5 (µL)**	**6 (µL)**	**7 (µL)**
E coli gDNA (100 ng/µL)	2	2	2	2	2	2	2
10X CutSmart Buffer[1]	2	2	2	2	2	2	2
*Rsa*I (10 U/µL)	1						
*Alu*I (10 U/µL)		1					
*Hpy*CH4V (5 U/µL)			2				
*Acc*II (10 U/µL)				1			
*Hae*III (10 U/µL)					1		
AccII + HaeIII[2]						1	
AluI + HypCH4V							1
Nuclease-free water	15	15	14	15	15	15	15
Total	**20**	**20**	**20**	**20**	**20**	**20**	**20**

[1] Use of CutSmart Buffer (NEB) is highly recommended. Buffers from other vendors have not been tested and may not be compatible with the restriction enzyme(s) used to digest the gDNA.

[2] If using a pool of restriction enzymes, mix 10 Units of each enzyme in a 1.5 mL LoBind tube and use 1 µL for the digest.

Appendix B. The Effect of the Size Range of Sheared DNA on Insert Size

When shearing DNA, it is important to create and size-select for fragments that are larger than the desired size range. Any given preparation of sheared DNA contains more fragments of smaller sizes (Panel A), which are cloned more efficiently than the larger fragments. Thus, the insert size of your final library will be skewed to the lower size range of your sheared DNA.

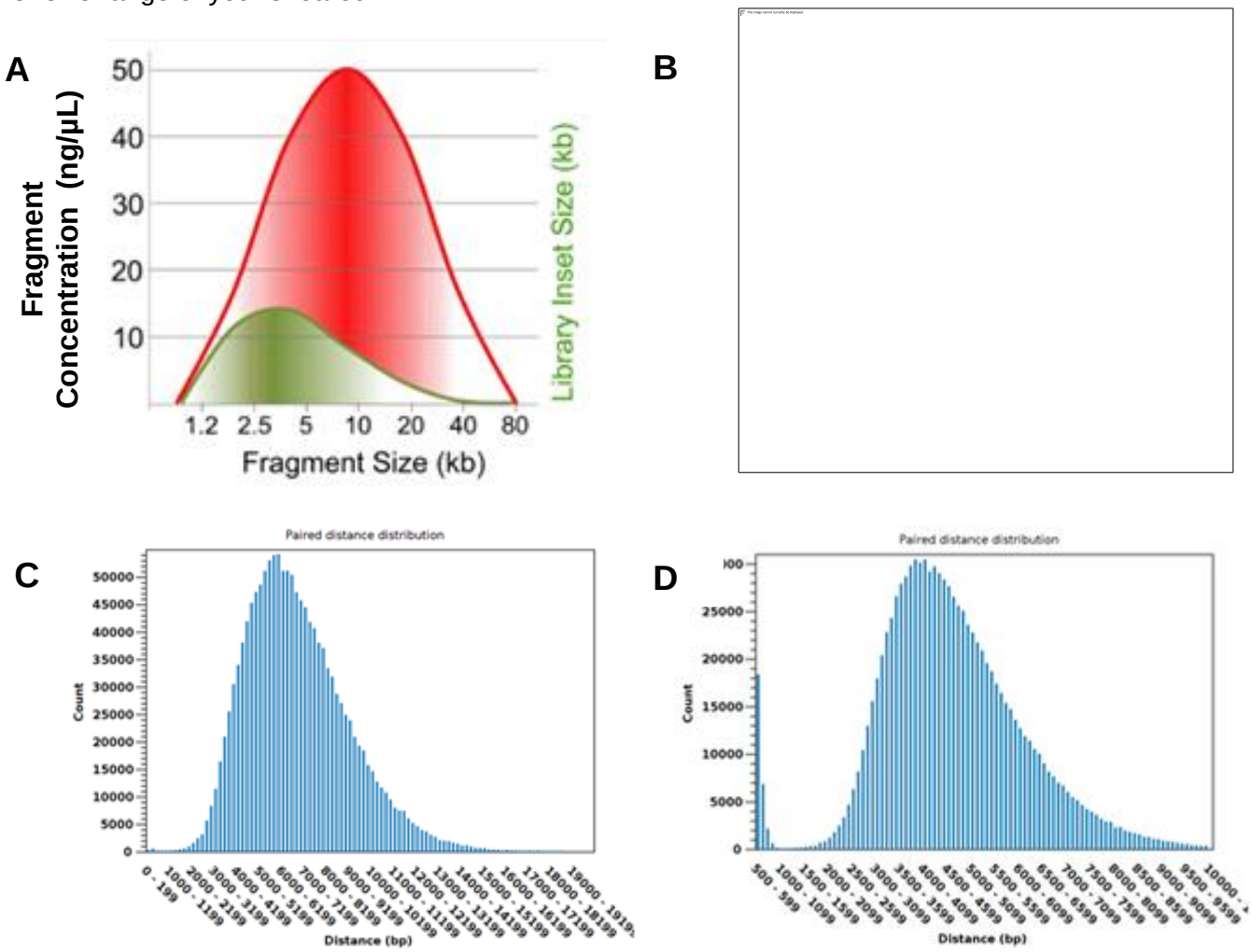


Figure 1: Effect of size range of sheared DNA on insert size. (A) The size range of fragments in sheared DNA is shown in the Red curve. A library created from this preparation of sheared DNA is typically skewed toward the lower end of the size range (Green curve) because of the number of molecules associated with a given size of DNA fragment (Given an equal mass of DNA, there will be more smaller fragments than larger fragments). (B) Examples of DNA sheared on a Megaruptor and with gTubes. Note that the final library size will be at the lower size range of the sheared DNA. (D and E) Analysis of two sequenced libraries: *T. aquaticus* gDNA (C), and *E. coli* (K12) gDNA (D), sheared with gTubes.

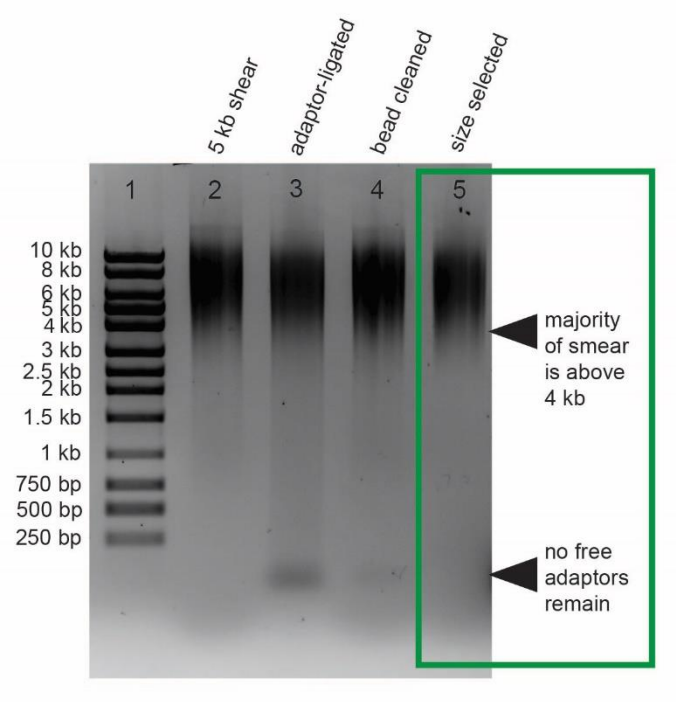


Figure 2. Confirmed size selection of 5 kb insert size in step 7.5. **Lane 2:** sheared genomic DNA from Step 1. **Lane 3:** End-repaired, A-tailed, adaptor-ligated insert. **Lane 4:** Bead-cleaned insert from Step 6. **Lane 5:** Size-selected insert ready for ligation to coupler. Check that no free adaptors remain in the sample (seen as a band below 250 bp) and that most of the smear below the desired insert size has been eliminated.

Appendix C. Determining Bead Concentration

The appropriate bead concentration is based on the lower end of the desired insert size range. For example, if your sheared material ranges in size from 2–4 kb, the amount of beads used in "Step 7: Size Selection" on page 17 should be 0.44X or 176 µL.

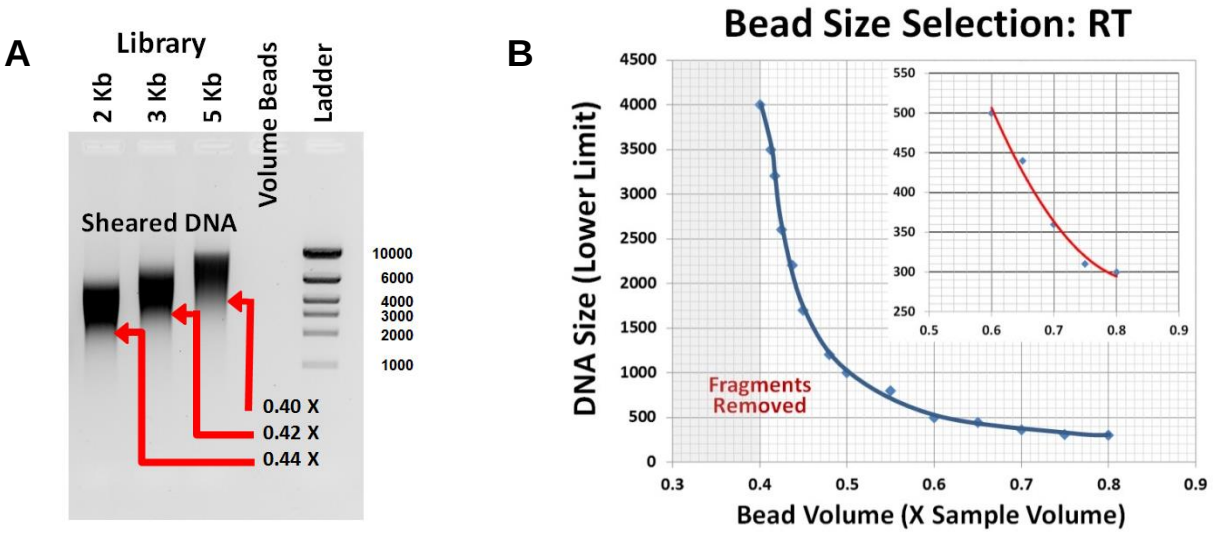


Figure 1: Effect of bead concentration on DNA size selection. (A), Gel image of three different insert sizes. The lower end of the size distribution is highlighted with the red arrows and the recommended volume of beads to be used is provided. (B) Comparison of bead concentrations to the sample cut off. When selecting the optimal bead volume for insert size selection, locate your insert size on the left axis and draw a horizontal line to the graphed line, and then draw a vertical line down to the bottom axis. Determine the bead volume and multiply this value by your sample volume. This value will be the amount of beads to add to your sample for size selection. Do not use less than 0.4X beads as this will result in the loss of your sample. The inserted graph in the upper right corner of graph B is an enlarged view of the smaller DNA fragments (300 to 500 bp). Beads must be brought to Room Temperature (RT) prior to use.

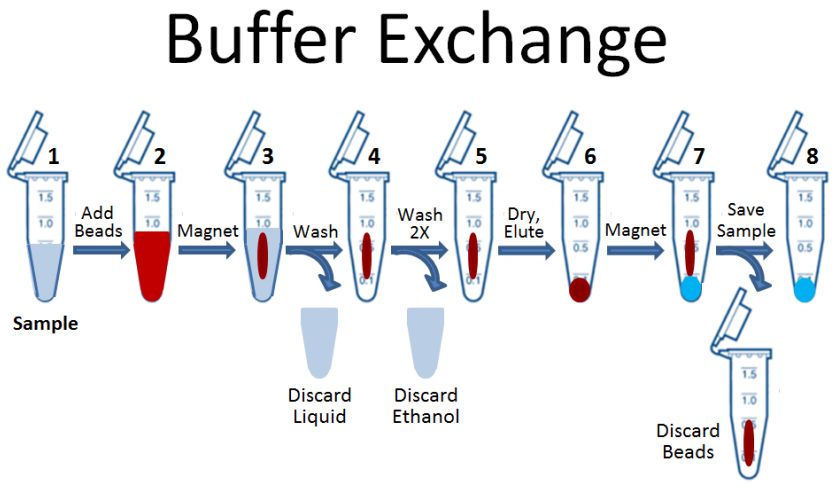


Figure 2. Buffer Exchange. 1) Sample DNA; 2) Add beads to sample and mix, incubate 5 min; 3) Place tube on magnetic rack; 4) Discard liquid and Wash 2x with 70% ethanol; 5) Dry beads for 5 min; 6) Add Elution Buffer, remove from magnetic rack to allow resuspension of DNA; 7) Place tube on magnetic rack after incubation; 8) Transfer liquid to new tube and discard beads.

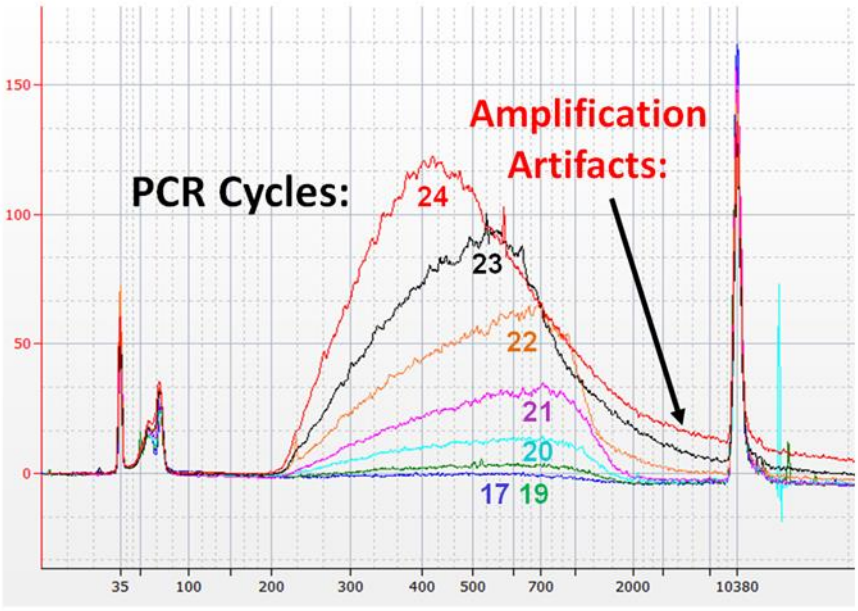
Appendix D: Additional Information on Amplification Artifacts

Amplification Artifacts:
Amplification artifacts are primarily due to limiting resources in the PCR master mix after several cycles of amplification.

Examples of amplification artifacts include:

- Single strand DNA which can form secondary structures (hairpins) and run at a higher size range (indicated by black arrow in figure below).
- Reduced amplicons size as shown in figure below for Cycles 23 (Black) and 24 (Red) for this GC-rich genome (78% GC).

Amplification artifacts can be reduced or eliminated by reducing the number of PCR cycles or increasing the post-PCR 72 °C extension time. For the example below, 20 or 21 cycles will produce sufficient DNA for library sequencing after 8 reactions are combined and size selected.



Over-Amplification
Over-amplification is defined as producing far more library DNA than is needed for sequencing. Over-amplification can be reduced or eliminated by reducing the number of PCR cycels used. In the example above, 20 or 21 cycels will produce sufficient DNA for library sequencing.

Appendix E: Additional Instructions for Sample Pooling Prior to Sequencing

Compatible Index Primer Combinations:
When using the Index Primer Mixes from the NxSeq® Long Mate Pair Index Kit**,** use the following guidelines to pool two or more indexed libraries together for single-indexed sequencing on an Illumina Sequencer:

Number of Pooled Samples	Options for Index Primers	Index Primers to Use
2	1	Index 6 and Index 12
3	1	Index 5, Index 6, and Index 12
	2	2-plex option with any other index
4	1	Index 4, Index 5, Index 6, and Index 12
	2	3-plex option with any other index

If pooling 5-12 samples, use the 4-plex options with any other available indices.

Libraries should be pooled in equimolar amounts. If multiplexing, normalize and pool samples according to the appropriate Illumina Platform User's Manual.

It is important to use compatible indices when pooling to maintain color balance of each base of the index read during sequencing. For proper imaging on the Illumina platform, at least one base needs to be read in the green laser channel (G or T) and the red laser channel (A or C). Using incompatible indices could result in image registration failure and ultimately run failure.

When setting up a sample sheet in Illumina Experiment Manager, select TruSeq LT from the drop down menu. Use A001-A012 when entering sample information, since these sequences correspond with the NxSeq Long Mate Pair Index sequences.

Appendix F: Double Bead Clean Up Protocol

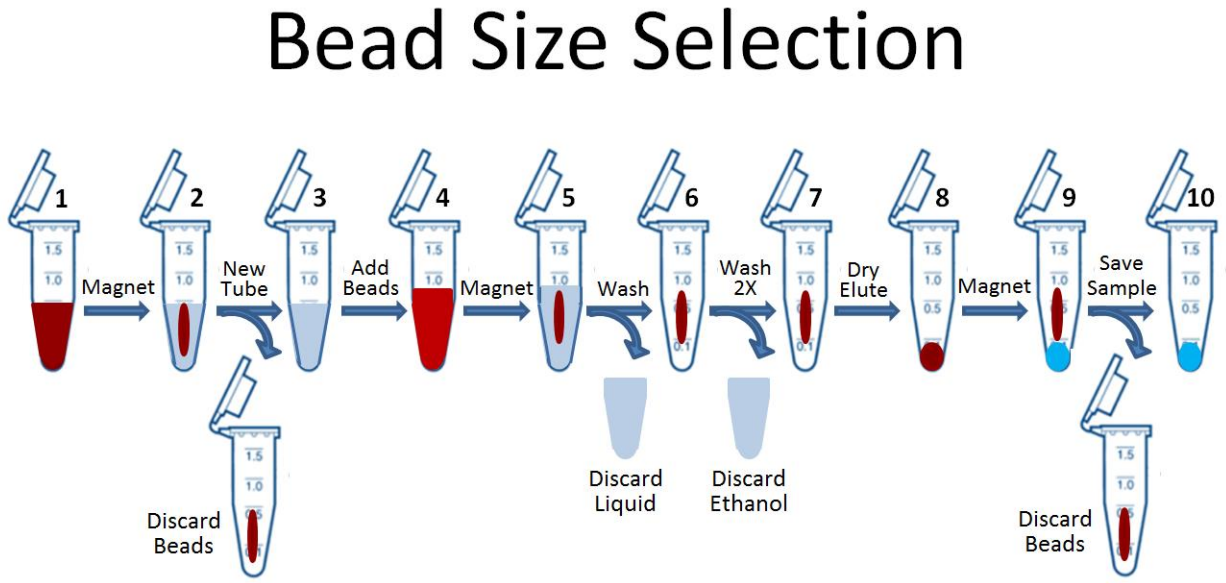


Figure 1. Double Size Selection. When a library contains fragments that are both too small and too large to sequence, these fragments can be removed with beads, resulting in a library with an optimal size range for sequencing. Briefly: 1) Add beads to sample and mix; 2) Place tube on magnetic rack; 3) Transfer liquid to new tube and discard tube with beads; 4) Add second volume of beads and mix; 5) Place on magnetic rack; 6) Discard liquid and Wash 2x with 70% ethanol; 7) Dry beads; 8) Add Elution Buffer and remove from magnetic rack to allow for resuspension of DNA; 9) Place tube on magnetic rack; 10) Transfer liquid to new tube and discard beads.

Bead size selection is based on the concentration of Polyethylene glycol (PEG) and sodium chloride (NaCl) in the bead buffer solution. A higher concentration will bind both small and large fragments while a lower concentration will only allow binding of larger fragments. When a small amount of beads and buffer is added to your sample, large DNA fragments will bind to the beads and when these beads are discarded, the large contaminating DNA fragments will be discarded with the beads. By adding a second aliquot of beads and buffer to your sample, the concentration of PEG and NaCl will increase and allow binding of the desired range of DNA fragments. Smaller, contaminating DNA fragments will not bind and will be removed when the beads are washed.

If your optimal library size range for sequencing is between 400 and 900 bp for example, you would add 0.515X beads and buffer to your sample based on the graph in Figure 1B in Appendix C. This amount of beads will bind fragments of 900 bp and larger for removal. When you add 0.155X beads and buffer to the saved supernatant, the buffer concentration will be increased to 0.67X beads and buffer (0.67X – 0.515X = 0.155X beads) and will bind DNA fragments between 400 and 900 bp. DNA fragments smaller than 400 bp will be removed when the beads are washed. (X = original sample volume).

Appendix G. Sequence Analysis: Filtering, Scripts, and Assembly Software Options

Location of Scripts

The scripts can be found at: http://www.lucigen.com/NGS-Long-Read-Mate-Pair-Scripts-Sample.html.

Overview

Sequence reads from Illumina instruments are output as two reads for each cluster, corresponding to the left and right ends (respectively) of the amplified molecule. Each read should begin with a Chimera Code™ and linker sequence. The Junction Code™ marks the location of the central portion of the original insert, and may occur in R1 only, in R2 only, or (for small inserts) in both R1 and R2. The workflow first filters the two raw sequence files (R1.fastqand R2. fastq) into four files:

• R1 and R2 of "mates" containing only true mates (matching Chimera Codes found at the beginning of both R1 and R2), and
• R1 and R2 of "nonmates" containing everything else, which will include chimeras (mismatched Chimera Code™ sequences) and non-mate reads (lacking a recognizable Chimera Code™).

The "mates" files must be split into simulated "Read1" and "Read2" files for use by assembly programs, by detecting the Junction Code and trimming appropriately. If the Junction Code is not found in either R1 or R2, both reads are saved in the 'unsplit_R1R2' file and may be used as single unpaired reads for assembly

Notes:

- The NxMate workflow results in mate pair reads that are in Forward-Reverse orientation (FR).
- IlluminaChimera-Clean4 script also trims the 5' end of every true mate read to remove the Chimera Code and linker sequences. Reads saved into the 'nonmates' file are not trimmed or otherwise processed.
- Contamination scanning and deduplication should be performed after splitting the reads to avoid influence of Chimera Code™ and Junction Code™ sequences.

Software requirements (tested on Ubuntu 12.04)

Software	Available From
Python 2.6 or 2.7	https://www.python.org/downloads/
ParseFastQ.py	https://gist.github.com/xguse/1866279
Regex module	https://pypi.python.org/pypi/regex
BioPython	http://biopython.org/wiki/Download
IlluminaChimera-Clean4.py	Lucigen
IlluminaNxSeqJunction-Split7.py	Lucigen

Perform Sequence Analysis

1. Copy IlluminaChimera-Clean4.py, IlluminaNxSeqJunction-Split7.py and ParseFastQ.py into directory with the uncompressed R1 and R2 fastq sequence files

2. Open terminal or command prompt and cd into the directory with sequence file and scripts, then type:
   python IlluminaChimera-Clean4.py your_R1_inputfilename.fastq

Note: the input filenames must contain 'R1' and 'R2'. During processing the script will use the R1 filename as the base filename.

Four output files will be created:
mates_ICC4_your_R1_filename.fastq
mates_ICC4_your_R2_filename.fastq
nonmates_ICC4_your_R1_filename.fastq
nonmates_ICC4_your R2_filename.fastq

3. Split the mates files into simulated Read1 and Read2 files by running JunctionSplit7.py. Type: python IlluminaNxSeqJunction-Split7.py mates_ICC4_your_R1_inputfilename.fastq.

   (Again, it is not necessary to specify the R2 filename if both names differ only at 'R1' vs 'R2').

   Three sequence output files will be created:
   R1_IJS7_mates_ICC4_your_R1_filename.fastq
   R2_IJS7_mates_ICC4_your_R2_filename.fastq
   unsplit_JS7_mates_ICC4_your_R1R2_filename.fastq

4. The R1 (F) and R2 (R) files can be loaded in an assembler program such as SPAdes or CLC Genomics Workbench as FR mate pairs.

5. For programs expecting RF pairs (e.g. DNAStar SeqMan NGen, etc), reverse complement both files with Seqtk or equivalent.

6. Specify the expected mate pair distance based on your initial fragment size selection during library construction. A reasonable range to begin with is +/- 20% of initial fragment size, e.g., for a 5 kb library, initially specify 4 kb - 6 kb.

Assembly Software Options:

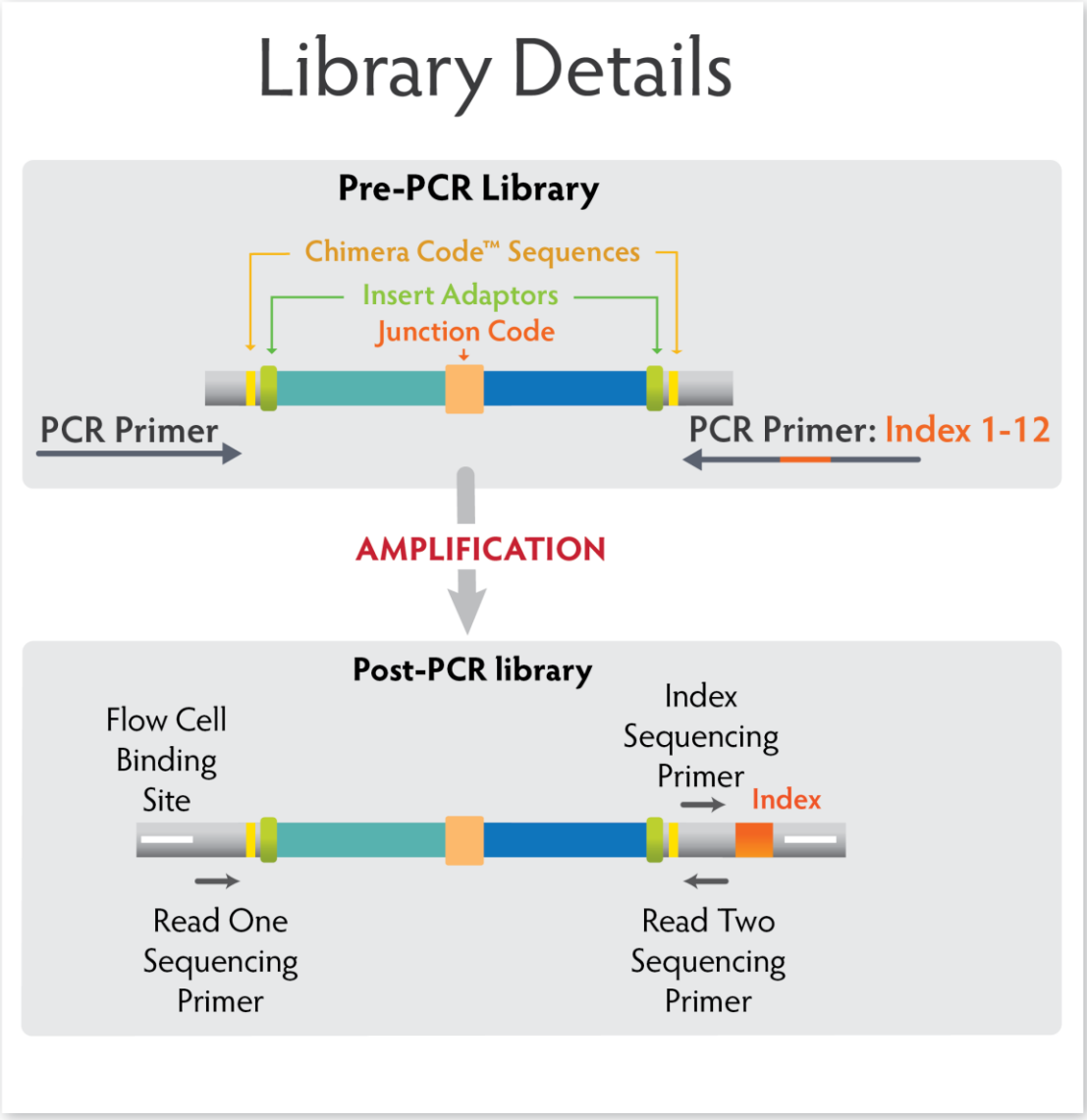
De novo sequencing provides novel information for a target with unknown sequence. However, sequencing without a reference poses challenges. Several analysis software options, listed in the table below, make *de novo* sequencing possible. The program SSPACE can be used to add mate pair data to existing assemblies/contig sets.

Open Source	Commercially available
Ray	DNAStar SeqMan NGen
Allpaths-LG	CLC Genomics Workbench
Velvet	SOFTGENETICS NextGENe
SPAdes 3.5*	
ABySS	

*Can use raw sequencer reads as input.

Appendix H: Sequence and Location Information of Adaptor, Chimera Code™ Sequences, and Junction Code™ Sequence

Feature	Sequence
Read 1 (left) Adaptor	5' CCACTGTGTCCGTCAAGCGAT 3'
Read 2 (right) Adaptor	5' CCAATGTGTCCGTCAAGCGAT 3'
Junction Code™ Sequence	GGTTCATCGTCAGGCCTGACGATGAACC



Chimera Code #	Sequence		Chimera Code #	Sequence
1	TGGACT		2	TCTGGA
3	ACTTCG		4	TGATGT
5	TGAGTC		6	TCGTGA
7	TGACTG		8	GTGCTA
9	TCAGGT		10	GAGGTT
11	ATGTCA		12	AGTTGT
13	GTATGA		14	TTAGAC
15	GTCTAC		16	CGTGTA
17	GTTGGA		18	GTTCTC
19	CGATTC		20	AATCTC
21	GGTTAC		22	TAGGTC
23	TCACCT		24	GAGTCT

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